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TNF- α BLOCKADE BY A DIMERIC TNF TYPE I RECEPTOR MOLECULE
SELECTIVELY INHIBITS ADAPTIVE IMMUNE RESPONSES

D. B. Colagiovanni^{1*}, M. A. Suniga¹, J. L. Frazier², C. K. Edwards, III¹, M.
Fleshner³, J. A. McCay⁴, K. L. White, Jr.⁴, and G. M. Shopp¹

¹Pharmacology Department, Amgen, Inc., One Amgen Way, Thousand Oaks, CA
91320

²Immunochemistry Laboratory, Amgen, Boulder, CO 80301

³Department of Psychology, University of Colorado-Boulder, Boulder, CO 80309

⁴Department of Pharmacology and Toxicology, Medical College of Virginia/Virginia
Commonwealth University, Richmond, VA 23298

ABSTRACT

Tumor necrosis factor- α (TNF- α) is a mediator of severe inflammatory processes, including rheumatoid arthritis. Suppression of TNF with a soluble type I or type II receptor molecule (TNF-RI or TNF-RII) has the potential to decrease cytokine levels and modulate inflammatory diseases in humans. However, it has recently been reported that treatment of mice with a TNF-RI:Fc immunoadhesin protein augmented Gram positive infections and subsequent mortality. To determine if TNF- α blockade with soluble TNF- α receptors might alter immune system function, assays were assessed in rodents treated with a dimeric form of the p55 TNF-RI, Tumor Necrosis Factor-binding protein (TNFbp). Administration of TNFbp resulted in suppression of primary and secondary IgG antibody responses and cell-mediated immune function. No treatment-related differences were detected in immune-enhancing assays or non-specific immune function parameters. Bacterial host resistance assays with *Listeria monocytogenes*, *Staphylococcus aureus* or *Escherichia*

coli showed an increase in tissue colony counts only with *L. monocytogenes* challenged animals following TNFbp administration. These results suggest that TNFbp has the capacity to inhibit adaptive immune function in experimental animal models. Studies suggest that while reducing TNF- α is important in controlling cytokine-dependent disease states, maintenance of a threshold level may be critical for normal immune function.

INTRODUCTION

Tumor necrosis factor- α (TNF- α)¹ appears to play critical roles in immune system function and frequently, dysfunction. A number of important regulatory functions in which TNF- α is involved, include T-cell proliferation, B-cell co-stimulation, augmentation of MHC class I and II expression and stimulation of other cytokines (1, 9, 27). TNF- α contributes also to non-specific inflammatory reactions by stimulating the acute phase response and activating vascular endothelium (4). Protection against bacterial and fungal infections including *Listeria monocytogenes*, *Pneumocystis carinii* and *Candida albicans* demonstrates an essential role for TNF- α (9, 24, 28, 33, 38).

While TNF- α is important for regulation of numerous inflammatory and immune system reactions, excessive production of this cytokine can create biological havoc (49). Inflammatory disease states such as rheumatoid arthritis (RA) and Crohn's Disease appear to be influenced by abnormally high TNF- α production (6, 17, 19). TNF- α and IL-1 β have been readily detected in the synovial fluid of RA patients (20) and several human trials have confirmed the usefulness of pro-inflammatory cytokine blockade (12, 16, 43, 44).

In light of the potential harmful effects that excessive TNF- α may cause in RA disease development, the use of cytokine inhibitors may alleviate some of the dysregulated overproduction. Modulation of inflammatory diseases through regulation of cytokine production and local cytokine levels has tremendous therapeutic potential. Cytokine inhibitors, such as interleukin-1 receptor antagonist

(IL-1ra) or a soluble form of type I TNF receptor, Tumor Necrosis Factor-binding protein or TNFbp, have been shown to limit disease severity in experimental animal models (15, 18, 37). Clinical trials have also yielded successful results with monoclonal antibodies to inflammatory cytokines by inhibiting disease pathology (16, 40). TNFbp was considered for clinical use in the treatment of inflammatory diseases. The molecule is composed of two soluble TNF receptor I subunits (P55) joined by a 20K polyethylene glycol (PEG) linker. It blocks the action of soluble and membrane bound TNF- α and TNF- β by binding directly to the ligand. While the intent of this drug therapy was to inhibit TNF- α induced pathology, blockade of TNF- α has potentially unintentional negative impacts on normal immune function and host resistance.

Reduction of pro-inflammatory cytokine responses can alter the normal protective mechanisms against invading pathogens. Inhibition of these cytokines has been shown to adversely influence host immune function. TNF- α or IL-1 β blockade, with specific antibodies or murine gene knockout strains, demonstrates increases in mortality in animal models of infection (26, 40). Animals challenged with Gram positive bacteria displayed inhibited granulocyte and monocyte influx to inflammatory sites following treatment with anti-TNF antibody (47). In addition, a TNF-RI:Fc immunoadhesin protein augmented Gram positive infections, including listeriosis, and subsequent mortality in murine studies (23). In the clinic, sepsis patients treated with a p75 sTNF-RII fusion protein construct experienced high mortality rates at the highest dose *versus* the placebo group (21).

Based on the hypothesis that cytokine blockade may impair host resistance and immune function, assays to evaluate cell-mediated immunity (CMI) humoral immunity and non-specific immunity were conducted. Studies assessed Gram positive and Gram negative bacterial challenge models; auto-immunity; antibody production, the reticulo-endothelial system, and contact sensitization (30). Overall, the findings suggest that TNF- α was critical to normal functioning of the immune system. In particular, humoral immunity and host resistance that are dependent of CMI were altered with TNFbp treatment.

METHODS

Animals

Female Balb/C, DBA/2, and C57BL/6 mice (18 - 25 grams) and adult female Sprague-Dawley (SD) rats weighing 140 to 170 grams were obtained from Charles River Laboratories (Portage, MI). Rodents were acclimatized for 7 to 10 days before initiation of experiments and maintained on food (Harlan Teklad Rodent Chow or Agway Rat Ratio (NIH 07) and sterilized water *ad libitum*. All animals were housed at the animal vivarium (Amgen Boulder), with the exception of SD rats used in the RES assay (Medical College of Virginia). All animals were ordered virus antibody free. For infection control procedures, cages with aspen chip bedding were isolated in a negatively pressurized room to prevent facility contamination. All animal use was in accordance with USDA guidelines for humane care of laboratory animals.

Recombinant Protein

Recombinant human soluble Type I TNF receptor was purified, sequenced and cloned at Amgen Boulder (25). A dimeric form was engineered by attaching a bifunctional 20,000-Da PEG molecule to a cysteine that was substituted by site-directed mutagenesis at residue 105. The resulting PEG-linked dimeric form, TNFbp, is a potent inhibitor of TNF (8, 42). TNFbp vehicle was formulated in a sorbitol solution (4%) with sodium chloride, 34 mM, and sodium phosphate, 10 mM at pH 6.5. Different molecular constructs of the p55 receptor have been considered for clinical use in the treatment of inflammatory diseases. An additional truncated, monomeric form of TNFRI containing the N-terminal 105 amino acids of the p55 receptor and a single PEGylated 30 KD molecule attached at the N-terminus (sTNF-RI) was also evaluated.

Antibody Response to Kevhole Limpet Hemocyanin (KLH)

TNFbp was administered as a single IV injection on day 0 at 0.4, 4 or 40 mg/kg to unanesthetized SD rats ($n = 9$). TNFbp treatment groups were compared to a vehicle control group, sTNF-RI at 0.4 and 4 mg/kg or a heat denatured form of TNFbp at 40 mg/kg. KLH from *megathura crenulata* (Calbiochem) was dissolved in sterile, room temperature physiologic saline (200 μ g/0.25 ml). KLH was injected IV on day 1 of the study. Antibody responses were detected from serum samples at pre-determined time points for IgM (days 5 - 21) or IgG (days 7 - 230) using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96 well plates were coated with 1 μ g/mL KLH and blocked with 2% BSA blocking buffer. Samples and controls were serially diluted from 1:200 - 1:3200 in 20% normal goat serum/plate wash buffer (PBS/ 0.05% Tween) and added to the plates. Sample addition was followed by a goat anti-rat alkaline phosphatase secondary antibody to detect captured antibodies. Color development was with a P-nitrophenylphosphate substrate system. A plate reader measured OD at 405-490 λ , a dual wavelength kinetic reading. Values in the linear range of the sample curve were used for calculations (generally 1:1600). Each sample was normalized as a proportion of a known positive sample, a standard transformation for data reported using ELISA (22).

Mouse Ear Swelling Test (MEST)

To evaluate contact sensitization, Balb/C mice ($n=15$) were sensitized to TNFbp by topical drug application to the abdominal region. Mice were anesthetized with Isoflurane (AErrane®, Ohmeda, Liberty Corner NJ) and the fur was chemically depilated (Nair, Carter-Wallace, NY) to enable thorough drug application. On day 0 of the study, mice were intradermally (ID) injected with complete Freund's Adjuvant (Sigma). On days one, three and five, 100 μ l of 5 mg/mL TNFbp diluted in ethanol (70%) was applied to the belly and allowed to dry prior to recovery from anesthesia. 0.5% Dinitrochlorobenzene (DNCB) in ethanol was used as a positive control.

Ethanol was used as a diluent and as the vehicle for the control group due to its ability to rapidly desiccate. On day ten of the study, the mice were challenged on the ear with the sensitizing agent. Mouse ear thickness was quantitatively measured (46) with calipers (Swiss Precision Instruments, NJ) and the coloration of the dorsal and ventral sides of the ear noted. At 24 and 48 hours post-application, the ears were again measured, color recorded and compared to the day's previous reading.

Mixed Lymphocyte Response (MLR)

To assess CMI, the lymphoproliferative response of spleen cells to allogeneic cells was evaluated (34). The *in vitro* unidirectional MLR assay was utilized as previously described (30), with modifications. DBA murine spleen cells, serving as the stimulator population, were excised (5×10^6 cells/mL) and treated with Mitomycin-C (Sigma) to suppress activity. The responder population of C57Bl/6 cells was cultured in a microtiter plate (2×10^6 cells/mL, 100 μ l/well) in the presence of the DBA splenocytes. The mitogen Concanavalin A (Sigma) was used as a stimulating agent for DBA controls. Cells were incubated for 3 days in the presence of TNFbp prior to being pulsed with ^3H -thymidine (1 μCi /well). Counts per minute (CPM) were measured on a Beckman scintillation counter 18 hours-post ^3H -thymidine addition.

Macrophage RES Study

This assay, as previously described (48) was conducted to determine the effects of TNFbp on the functional activity of the reticuloendothelial system. Briefly, macrophages of the RES, including Kupffer cells of the liver and interstitial macrophages of the lung, are involved in removing and reprocessing non-functional

erythrocytes, leukocytes and platelets. Circulation and phagocytosis of ^{51}Cr -labeled sheep red blood cells in liver, spleen, thymus, kidney and lung were examined. SD rats ($n=8$) were treated one hour prior to the assay with a single IV injection of TNFbp. Three doses of TNFbp, 0.4, 4 and 40 mg/kg, were administered IV one hour prior to assay. A positive control, maleic vinyl ether (MVE) (Hercules, Inc.) was injected 24 hours prior to the assay. MVE causes a suppression of hepatic phagocyte function resulting in a decrease in blood clearance and liver uptake of sheep red blood cells.

Popliteal Lymph Node (PLN Assay)

The PLN assay looks at the potential of an agent to induce an autoimmune response (13). Injection of chemicals into the hind foot-pads of mice can cause hypertrophy, hyperplasia and/or a CD4/CD8 ratio change in the popliteal lymph node (14). C57/B16 mice ($n=5/\text{group}$) were injected with 50 μL of test article into each footpads. TNFbp at 5, 10 and 20 mg/Kg or TNFbp vehicle was i.d. injected into the right footpad of mice. Heat aggregated BSA was used as a positive control at 20 mg/kg. Animals were sacrificed 8 days post-drug administration and PLN excised. The nodes were weighed and then processed to a single cell suspension and cells enumerated.

Bacterial Preparation for Host Resistance Assays

In order to determine if TNF- α blockade by TNFbp might adversely impact a host's ability to protect against microbial pathogens, three bacterial infection models were examined in rats. TNF- α plays an essential role in host defense during primary *Listeria* infections (36). *S. aureus* infections were studied due to their

prevalence in infections of immunocompromised individuals. The Gram negative bacterium *E. coli* was selected to complement the Gram positive strains. Bacterial strains were purchased from American Type Culture Collection (Rockville, MD) *Staphylococcus aureus* (ATCC #27217); *Listeria monocytogenes* (ATCC #13932); *Escherichia coli* (ATCC #25922). Stock cultures were established and stored at -70°C in 1 mL aliquots in physiologic saline with 5% glycerol. One day prior to bacterial injections into rats, a single vial was quick thawed and the bacteria were reconstituted with trypticase soy broth. Bacteria were incubated overnight in a baffled flask at 37°C. Sixteen to 20 hours post-incubation, the bacteria were washed twice and resuspended in normal saline. The concentrations of bacteria were estimated on a spectrophotometer at 600 λ . The absorbance was compared to a standard curve that was previously generated. Actual counts injected into rats were confirmed by plating the final preparation of bacteria at serial dilutions on trypticase soy agar (Microbio, Denver, CO). SD rats (n = 8/group) were weighed and randomized into treatment or control groups. TNFbp at 2.5 and 25 mg/kg or vehicle was IV administered one hour prior to bacteria injections. IV administration of *L. monocytogenes*, *S. aureus* or *E. coli* was conducted by tail vein injection. The standard concentration used for IV injections was 10^7 colony forming units (CFU)/mL. The CFU/mL in the blood, liver and spleen were compared between TNFbp treated and control rats.

Tissue Processing

Blood, splenic and hepatic tissues were removed from SD rats on days 1 - 3 for bacterial isolation. Tissues were aseptically removed and weighed prior to homogenization with an Ultra-turrax T25 tissue homogenizer (Janke & Kunkel, IKA Labortechnik). Blood samples and homogenized tissues were plated with limiting dilutions on trypticase soy agar (Microbio, Denver, CO). Colony counts were determined 24 hours-post incubation by manually counting individual colonies.

Statistical Analysis

Data were analyzed using the Statview Program for Macintosh. For normally distributed data, one-way ANOVA with the Bonferroni/Dunn's Post-hoc test for pairwise comparisons was utilized. The data were presented as the mean \pm SEM. A Student's t-test was used for analyzing the MLR assay. Homogeneity was evaluated using the Bartlett's Test for homogeneity. ANOVA was used to evaluate homogeneous data and when significant differences occurred, the treated groups were compared to the vehicle control using the Dunnett's t-Test. The positive control was compared to the vehicle using the Student's t Test. For non-homogeneous data in the RES assay; a non-parametric ANOVA was conducted and treated groups were compared to the vehicle control group using the Wilcoxon Rank Test when significant differences occurred. For the host-resistance studies, the Kruskal-Wallis one-way ANOVA on Ranks was used due to the non-normality of these data. Pairwise comparisons were conducted using the Dunn's method. Data were presented as the median with 25th percentile (25p) and 75th percentile (75p) values reported.

RESULTS

Humoral immune function (KLH)

To determine if drug therapy might impair antibody production to a foreign antigen, rats were challenged with KLH following TNFbp administration. The IgM response, measured from days 5 - 21, was unaffected by administration of .4 - 40 mg/kg TNFbp (data not shown). TNFbp, however, caused a reduction in anti-KLH IgG antibody production. At doses of 4 and 40 mg/kg TNFbp, KLH antibody generation was significantly reduced (FIG.1A). In the primary response, all TNFbp dose groups were initially suppressed to a similar degree, however by day 40 post-

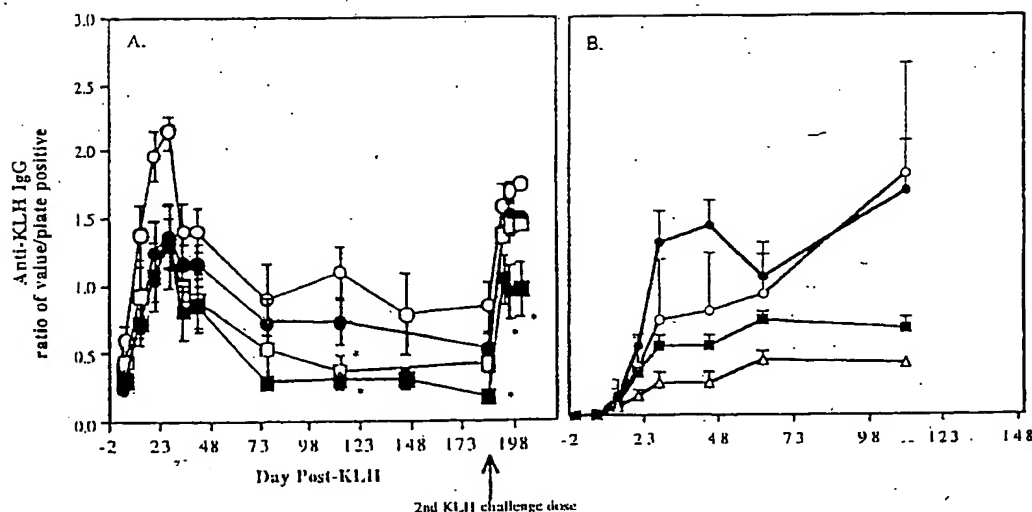


FIG 1. Production of anti-KLH IgG antibody in Sprague-Dawley Rats. On day 0, rats were IV administered drug or vehicle. On day 1, animals were IV challenged with KLH (200 μ g/250 μ l). On days 0 and 5, TNFbp treated rats were IV administered drug. ○ = vehicle control; ● = 0.4 mg/kg TNFbp; □ = 4 mg/kg TNFbp; ■ = 40 mg/kg TNFbp. 1B. ○ = vehicle control; ● = 0.4 mg/kg sTNF-R1; □ = 4 mg/kg sTNF-R1; ■ = 40 mg/kg heat inactivated sTNF-R1. Serum KLH antibody values were measured by ELISA and represent the mean of 9 rats \pm SEM. * = statistically significant from control group ($P < 0.05$).

KLH; a dose-response relationship was evident. At 115 days post-KLH no differences from control animals occurred in the 0.4 mg/kg TNFbp treated animals, while a significant decrease in antibody production occurred with 4 and 40 mg/kg TNFbp treated animals. This suppressed KLH antibody response persisted until day 183. The heat denatured TNFbp administered at 40 mg/kg, which served as an inactive protein control, did not alter KLH IgG response compared to the control group. Following a second administration of KLH on day 190, the high dose TNFbp treatment group (40 mg/kg) caused a significant suppression of antibody responses on days 193 and 202. This suppression of antibody occurred without re-administration of TNFbp, suggesting an impact from the drug therapy 190 days prior.

While KLH IgM and IgG antibodies were measured, serum antibodies to TNFbp were also examined. The question arose as to the impact of anti-TNFbp antibodies on the generation of anti-KLH antibodies. Use of the recombinant human

TNFbp in rats did result in sustained anti-TNFbp IgG antibody formation (titers > 1:100,000, data not shown). However, a non-immunogenic formulation of TNFbp also inhibited KLH antibody generation (data not shown). sTNFRI was only mildly immunogenic (titer < 1:1000). In this experiment, sTNFRI also inhibited KLH antibody generation. Administration of sTNF-RI at 0.4 or 4 mg/kg decreased anti-KLH responses in comparison to control animals (FIG. 1B), with effects out to 112 days. The heat denatured sTNF-RI administered at 40 mg/kg, which served as an inactive protein control, did not alter KLH IgG responses compared to the vehicle control group. These results suggest that antibody production to the TNFbp protein molecule is not responsible for the inhibited KLH antibody responses.

Cell-mediated immune function (MEST and MLR)

In vitro and *in vivo* models of CMI were examined to determine if TNFbp might impair T-cell dependent immunity. The MEST evaluated TNFbp *in vivo* for the induction of a delayed type hypersensitivity reaction. The MEST measured type IV, T-cell mediated antigen specific contact sensitivity reactions (46). Development of erythema of the ear occurred in response to antigen recruitment of macrophages, basophils, and eosinophils with mediator release. In this assay, generation of an immune response may indicate that a compound is "immuno-enhancing". In contrast to the positive control response with DNCB, following challenge with TNFbp there were no differences in ear thickness compared to ethanol or complete Freund's Adjuvant control groups (FIG. 2).

In the second assay for CMI, the MLR, *in vitro* cellular proliferation was correlated with ^3H -thymidine incorporation. The lymphoproliferative response of spleen cells to allogeneic cells is a sensitive indicator of CMI (34). This *in vitro* assay examined splenocyte proliferation with TNFbp added directly to the culture wells. A significant decrease in ^3H -thymidine incorporation was evident in the 11 $\mu\text{g/mL}$ TNFbp dose group compared to the vehicle group, while 0.11 and 1.1 $\mu\text{g/mL}$ treatment groups were equivalent to control (TABLE 1). The decrease represented a 35% reduction in response.

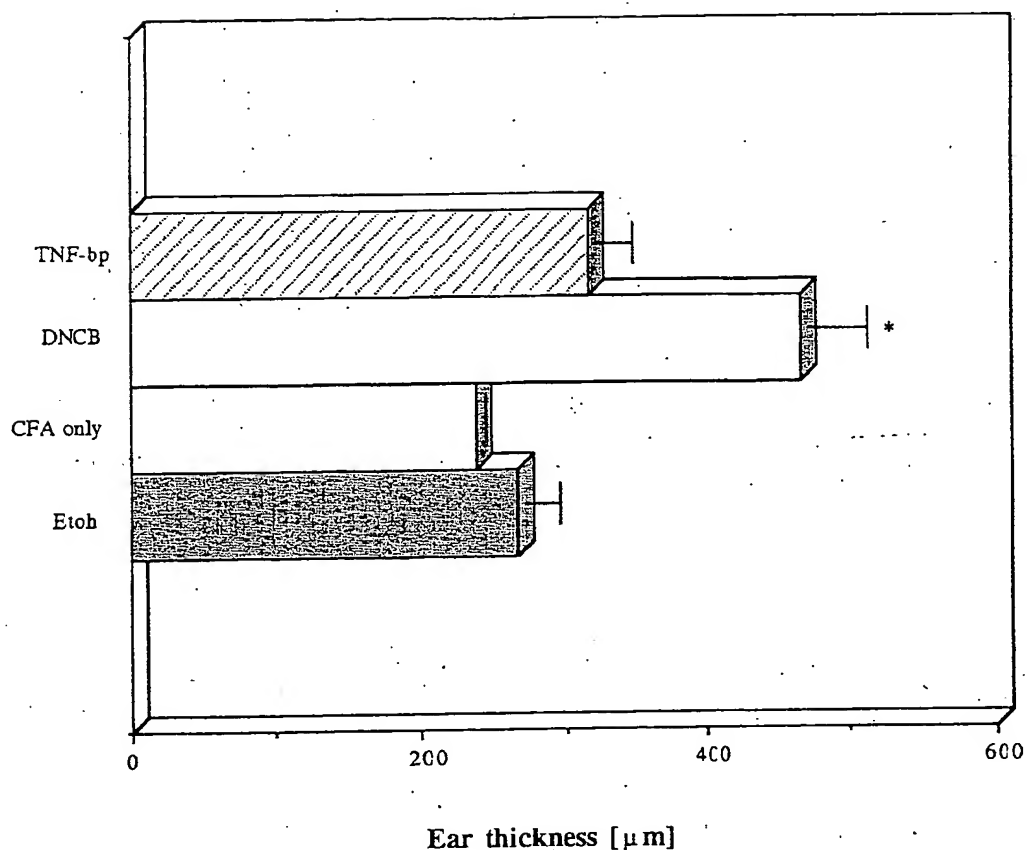


FIG. 2. Mouse Ear Swelling Study. Evaluation of ear thickness in Balb/C mice after drug challenge. Ethanol was the drug diluent and served for a vehicle control group. 0.5% DNCB was the positive control group. TNFbp was tested at 5 mg/mL. Ear challenge occurred on day 10 post-i.d. CFA. Twenty-four hours post-ear challenge, caliper measurements were conducted and erythema noted. Values represent the mean ear thickness of 15 mice \pm SEM. * = significantly different from control group ($P < 0.05$).

Non-specific immune function (RES and PLN Assays)

The RES assay examined functional changes of acute drug treatment. Treatment with TNFbp did not cause significant changes in the functional activity of the RES as measured by vascular clearance rates or the phagocytic uptake in ^{51}CR -labeled sheep red blood cells in the liver, spleen, thymus, lungs or kidneys (TABLE 2). The positive control, maleic vinyl ether, produced a major decrease in hepatic phagocytosis of 73% and increased half-life clearance of 194%.

TABLE 1

Mouse^a Mixed Lymphocyte Response (MLR)

TNFbp <i>in vitro</i> (μ g/mL)	Mean cpm ^b ($\times 10^{-3}$) \pm SEM
0	25.1 \pm 1.4
0.11	22.9 \pm 2.9
1.1	25.7 \pm 1.3
11	^c 16.4 \pm 2.5

^a-Data are the mean values (\pm SEM) for splenocytes from 4 mice in one of three experiments with similar results. Cells were incubated in the presence of TNFbp for 3 days prior to being pulsed with ³H-thymidine. Counts were measured 18 hours post-³H-thymidine addition.

^b-Net cpm of stimulated cultures are determined by subtracting the cpm of responder cell only cultures from the cpm of responder + stimulated cell cultures.

^c-Treatment significantly different from the vehicle group ($P < 0.03$) by Student's T-test.

To rule out a role for TNF- α in the induction of autoimmune disease processes, the PLN was used to evaluate immuno-enhancing agents capable of inducing autoimmune-like reactions (14). No effects on autoimmune responses with TNFbp treatment were demonstrated in the PLN assay (FIG. 3). In this study, popliteal lymph nodes were excised, weighed and enumerated. At the three doses evaluated, there were no significant differences ($P > 0.05$) from the vehicle control group. The positive control, heat aggregated BSA, caused a dramatic increase in the size of the nodes (+400%). Accordingly, cellularity was also increased in this treatment group. No changes in CD4/CD8 cellular population dynamics were detected by flow cytometry with TNFbp or BSA (data not shown).

Host resistance assays

The capacity of rats to suppress a systemic infection with the aerobic bacterial strains *Listeria monocytogenes*, *S. aureus*, and *E. coli* was evaluated. The bacterial challenges were evaluated on day 3 post-injections. Blood, liver and spleen samples

TABLE 2

Functional Activity of the Reticuloendothelial System in Female Sprague Dawley Rats Treated with TNF- α

Parameter	Vehicle (10)	0.4 mg/kg (10)	TNF- α 4.0 mg/kg (10)	40 mg/kg (10)	MVE 50 mg/kg (8)	HNH
Vascular Half-Life 51Cr sRBC	14.2 \pm 3.6	17.6 \pm 2.1 ^b	13.3 \pm 1.4 ^a	14.8 \pm 2.1 ^b	41.7 \pm 5.5 ^{**}	H
Body Wgt (g)	215.7 \pm 4.5	212.7 \pm 4.6	213.6 \pm 4.5	214.1 \pm 2.8	199.9 \pm 4.8 [*]	H
Liver Wgt (mg)	10066 \pm 406	9601 \pm 408	9628 \pm 380	9454 \pm 390	9732 \pm 412	H
% Uptake	54.6 \pm 4.1	46.4 \pm 4.2	51.2 \pm 3.7	50.4 \pm 3.4	14.5 \pm 1.5 ^{**}	H
cpm/mg	383 \pm 26	340 \pm 31	372 \pm 28	380 \pm 24	98 \pm 9 ^{**}	H
Spleen Wgt (mg)	495 \pm 27	486 \pm 26	480 \pm 27	509 \pm 21	535 \pm 19	H
% Uptake	6.1 \pm 0.5	5.7 \pm 0.5	5.9 \pm 0.6	6.0 \pm 0.7	7.2 \pm 1.5	H
cpm/mg	888 \pm 68	820 \pm 54	843 \pm 102	846 \pm 117	890 \pm 188	H
Lung Wgt (mg)	1367 \pm 65	1511 \pm 153	1494 \pm 92	1416 \pm 54	1435 \pm 89	NH
% Uptake	0.33 \pm 0.07	1.00 \pm 0.46	0.34 \pm 0.04	0.42 \pm 0.08	2.69 \pm 0.36 ^{**}	NH
cpm/mg	18 \pm 4	50 \pm 26	16 \pm 2	20 \pm 4	127 \pm 18 ^{**}	NH
Thymus Wgt (mg)	578 \pm 30	515 \pm 29	554 \pm 46	519 \pm 32	472 \pm 35 [*]	H
% Uptake	0.021 \pm 0.004	0.038 \pm 0.007 [*]	0.023 \pm 0.003	0.028 \pm 0.004	0.055 \pm 0.007 ^{**}	H
cpm/mg	2.6 \pm 0.6	5.5 \pm 1.4 [*]	2.8 \pm 0.3	3.9 \pm 0.6	7.7 \pm 0.9 ^{**}	NH
Kidney Wgt (mg)	1800 \pm 61	1833 \pm 65	1827 \pm 64	1775 \pm 42	2008 \pm 85	H
% Uptake	3.0 \pm 0.3	4.1 \pm 0.4	3.4 \pm 0.2	3.6 \pm 0.3	2.8 \pm 0.6	H
cpm/mg	121 \pm 16	157 \pm 16	131 \pm 10	141 \pm 13	89 \pm 18	H

Female Sprague Dawley rats were administered vehicle or TNF- α i.v. one hour prior to assay. The positive control, MVE, was administered i.v. on day 1, the rats were evaluated for phagocytic activity by measuring the vascular clearance rate and organ distribution of 51Cr sRBC. Values represent the mean \pm SE derived from the number of animals indicated in parentheses. Values significantly different from vehicle control at $p < 0.05$ are indicated by an asterisk, while those significant at $p < 0.01$ are noted by a double asterisk. ^aN = 9; ^bN = 8

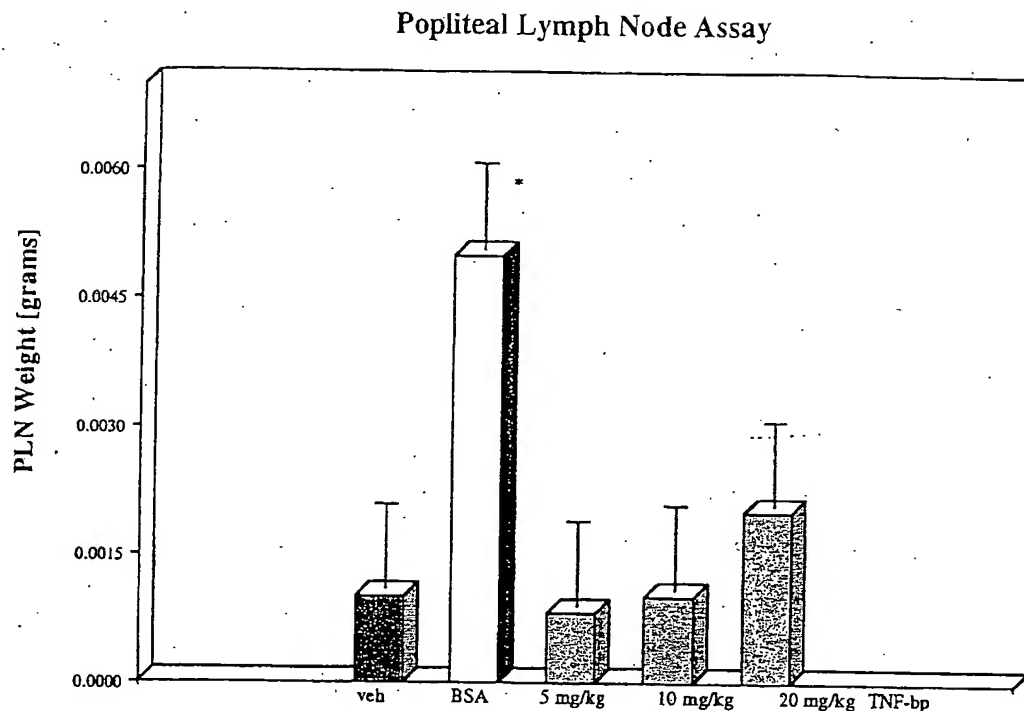


FIG.3. PLN Assay. C57Bl/6 mice were injected in footpads on day 1 of study with TNFbp, heat aggregated BSA, or vehicle. On day 8, PLN were excised and node weights determined. Values represent the mean weights of 5 animals \pm SEM. * = significantly different from control group ($P < 0.05$).

were assess for CFU/mL following 0, 2.5, or 25 mg/kg TNFbp administration (TABLE 3). No significant differences from control values in any organ were apparent in the 2.5 mg/kg treated groups with any bacterial strain evaluated. In the *S. aureus* and *E. coli* bacteremia models, no adverse effects of TNFbp were evident on liver, spleen or blood CFU/mL. The 25 mg/kg TNFbp dose caused slight increases in *E. coli* counts, however, the numbers were not significantly different from controls. Significant differences from control CFU/mL with *L. monocytogenes* were evident on day 3 for splenic tissue and blood cultures at 25 mg/kg dosing. Increases in CFU represented up to 100-fold greater bacterial seeding than in the control animals. A trend was also evident in the hepatic samples, but the numbers were not statistically significant.

TABLE 3

Host Resistance Assays in Sprague-Dawley Rats^a Three Days Post-Bacterial Challenge

Bacteria	Organ	TNF-bp dose (mg/kg)	CFU/ml	25p - 75p
<i>L. monocytogenes</i>	blood	0	3.3×10^2	2.0 - 8.8 ($\times 10^2$)
		2.5	1.7×10^3	.85 - 6.5 ($\times 10^3$)
		25	^b 1.44×10^4	.004 - 1.2 ($\times 10^5$)
	liver	0	3.4×10^5	.8 - 9.5 ($\times 10^5$)
		2.5	1.9×10^5	.06 - 1.2 ($\times 10^6$)
		25	8.1×10^6	.01 - 5.8 ($\times 10^7$)
	spleen	0	3.4×10^4	.21 - 5.8 ($\times 10^4$)
		2.5	6.8×10^5	.28 - 1.3 ($\times 10^6$)
		25	^b 1.6×10^6	.60 - 2.3 ($\times 10^6$)
<i>S. aureus</i>	blood	0	8.8×10^2	.55 - 5.7 ($\times 10^3$)
		2.5	5.7×10^3	2.7 - 9.4 ($\times 10^3$)
		25	2.8×10^3	2.0 - 7.4 ($\times 10^3$)
	liver	0	1.6×10^4	1.0 - 3.2 ($\times 10^4$)
		2.5	2.9×10^4	2.0 - 4.4 ($\times 10^4$)
		25	5.2×10^4	1.9 - 9.1 ($\times 10^4$)
	spleen	0	4.0×10^4	1.9 - 8.5 ($\times 10^4$)
		2.5	7.2×10^4	.36 - 1.4 ($\times 10^5$)
		25	1.1×10^5	.5 - 1.8 ($\times 10^5$)
<i>E. coli</i>	blood	0	1.3×10^4	.89 - 4.4 ($\times 10^4$)
		2.5	1.1×10^4	.02 - 1.2 ($\times 10^5$)
		25	3.1×10^4	1.3 - 4.8 ($\times 10^4$)
	liver	0	6.0×10^3	.11 - 2.5 ($\times 10^4$)
		2.5	2.9×10^3	1.5 - 4.9 ($\times 10^3$)
		25	5.8×10^3	1.3 - 9.6 ($\times 10^3$)
	spleen	0	1.1×10^4	.46 - 5.7 ($\times 10^4$)
		2.5	1.5×10^4	1.2 - 2.4 ($\times 10^4$)
		25	2.5×10^4	1.5 - 5.8 ($\times 10^4$)

^a Data are the median values (+ 25 - 75 percentiles) for 8 rats in one of four experiments with similar results.

^b $P < 0.001$ versus vehicle treated animals by Dunn's method of analysis.

DISCUSSION

Cytokine overproduction by local cellular populations can manifest as disease. For example, mast cells that localize near micro-vascular endothelium exacerbate inflammation in RA by producing cytokines, such as IL-1 and TNF- α (15, 49). In light of the potential harmful effects TNF- α may cause in RA disease development, the use of TNFbp or a similarly designed molecule may alleviate some of the dysregulated overproduction of this cytokine. A relevant question we posed in these studies was whether anti-TNF treatment compromised the host's immune system. Studies by other investigators have indicated TNF inhibition can adversely influence host immune function (7, 9, 29, 40). Through evaluation of a series of assays, TNFbp treatment was shown to selectively suppress cell-mediated and humoral immune function.

One of the most important findings of this study was the suppression of IgG antibody production with high dose TNFbp treatment. Measurement of the anti-KLH antibody response allows one to assess across time the development of *in vivo* humoral immune responses (31). This measure of humoral immunity has been used by others to study known immunomodulatory factors. For example, morphine decreases primary KLH antibody production following antigen challenge (32). Morphine administration also increases the occurrence rate of infectious disease (41). Thus, suppression in the *in vivo* anti-KLH IgG response is indicative of suppressed immunity. In the 40 mg/kg dose group, IgG suppression was sustained throughout the primary and secondary response after challenge with KLH. Although antibody generation occurred in these rats, we are uncertain if the decreased responses are significant enough to result in impaired protection against foreign antigens. Recent studies suggest that alterations in responses from control values of 15 - 25% are sufficient to cause a change in immunocompetence (36). We found antibody responses to be decreased up to 76% at some timepoints.

The decreased secondary antibody responses associated with TNFbp treatment were possibly due to a reduction in long-term memory cell production. A TNF-dependent component to B-cell antibody generation seems to be evident. Other

investigators have shown TNF- α to induce B cell proliferation and T helper cells that regulate B cell antibody production (27, 31). Recent studies have also shown a role for TNF in B-cell migration (10). Whether the blockade of TNF's action altered B-cell production or migration in the current study cannot be further elucidated.

In addition to its influence on antibody generation, TNF- α also was shown to be essential for effective CMI. Assays for CMI can detect whether effector cell function is altered (7, 35). A unidirectional MLR assay evaluated cellular proliferation by ^3H -thymidine incorporation. This *in vitro* assay examined splenocyte proliferation with TNFbp added directly to the culture wells. Decreased lymphocyte proliferation was evident, indicative of an effect on cellular function. The MLR was negatively impacted by TNFbp at 11 $\mu\text{g/mL}$. This decrease in proliferation only occurred in cells treated with the high dose of TNFbp, while lower doses tested were unchanged by drug treatment.

Host defense mechanisms mediated by CMI may also be affected by cytokine inhibition. Along with the MLR, alteration of host resistance is a relevant endpoint to examine immune dysfunction (5). In *L. monocytogenes* infections, which require a Th₁-driven CMI response for bacterial clearance, TNF- α is a partial mediator (11). The *L. monocytogenes* host resistance assay was altered by TNFbp treatment, leading to increased spleen and blood CFU/mL. Results indicated that blockade of TNF- α lead to an increase in systemic bacterial infection. In support of our findings, others have shown anti-TNF antibodies increase proliferation of *L. monocytogenes* in the spleen and liver in sublethally infected mice (28, 47). Additionally, studies utilizing p55 receptor knockout mice have demonstrated an increased susceptibility to listeriosis (40). The *Listeria* host resistance study and the MLR assay imply that high doses of TNFbp impair CMI. Animal models evaluating the anti-inflammatory potential of TNFbp have shown efficacy at 1-3 mg/kg dosing (personal communication, Alison Bendele). These anti-inflammatory doses are considerably lower than those used in the current experiments, indicating that a significant safety factor exists between efficacy and toxicity.

In contrast to the effects on host resistance with *Listeria*, anti-TNF therapies did not affect *S. aureus* or *E. coli* infections. These findings are consistent with

previous studies that showed treatment with anti-TNF therapies to diminish mortality after injection of *E. coli* or lipopolysaccharide (18, 39, 40). The primary defense mechanisms for *S. aureus* are polymorphonucleocytes (PMNs) and bacterial killing, while *E. coli* virulence is correlated with resistance to phagocytosis by macrophages and PMNs (5, 8). TNF- α is a central mediator of the lethal consequences of *Escherichia coli* infection. In these bacterial models, anti-TNF therapies may play a role in protection against infection.

As the results demonstrated, TNFbp at high doses caused immunosuppression in humoral and cell-mediated assays. Blockade of the action of TNF- α did not, however, cause immuno-enhancement. Both the MEST and the PLN assays were unaffected by TNFbp treatment. The MEST, a type IV, T-cell mediated antigen specific contact sensitivity reaction (46), measured TNFbp 24 - 48 hours post-challenge. Erythema with accompanying edema occurred in response to antigen recruitment of macrophages, basophils, and eosinophils with mediator release. In this assay, generation of an immune response may indicate that a compound is "immuno-enhancing". In contrast to the positive reaction with DNCB, no changes from the control group were evident following TNFbp treatment. The PLN assay, capable of detecting substances that can induce autoimmune-like conditions such as lupus syndrome, scleroderma, and generalized lymphadenopathy (14), was also not affected by TNFbp treatment. This assay has demonstrated sensitivity with other agents, such as the drug Zimeldine, which was withdrawn due to immune-mediated side effects (45). The findings are consistent with the immune function and host resistance results that demonstrate immunosuppression, but not immuno-enhancement, with TNFbp.

No effects on non-specific immunity were detected by examination of RES function using TNFbp treatment in an acute exposure model. The macrophages of the RES are critical to proper immune function, as they serve as a first line of defense for blood borne pathogens (48). RES analysis examined fixed macrophage function in rats as measured by the vascular clearance and organ uptake of ^{51}Cr -sheep red blood cells. This assay was important for demonstrating that macrophage function was not inhibited by TNF- α blockade. This result may initially seem to contradict the findings in the *Listeria* host resistance study, as macrophages are a central component

of protection against this pathogen. However, we speculate that TNF inhibition is affecting host resistance to this bacterium through a T-cell dependent mechanism, rather than directly affecting macrophages. Effective elimination of *Listeria* from host cells relies on T cell activation (28, 11). T cell deficient mice develop a chronic *Listeria* infection following challenge (2). These data are in agreement with the MLR results where a decrease in T-cell proliferation was exhibited following TNFbp exposure.

In these studies, we postulated that TNF- α is critical to memory B-cell generation and cell-mediated immune function, and a potential adverse effect of high dose therapy with TNFbp in animal models is immune system suppression. As noted previously, modest impairments of multiple components of the immune system might cause cumulative effects on the host (5). While reduction of TNF- α levels is the ultimate goal for anti-TNF therapy, some residual amount of this cytokine must remain locally for effective host resistance. Studies have indicated that TNF inhibition can adversely alter host immune function (7, 29). As others have suggested (3), complete ablation of TNF- α may have negative impacts on the immune system. A balance must be achieved whereby a minimal TNF concentration is maintained *in vivo*, while excessive production of this cytokine is thwarted. The complex nature of TNF regulation in health and disease offers an opportunity for many individuals committed to understanding the processes of inflammation.

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FOOTNOTES

Abbreviations: tumor necrosis factor- α , TNF- α ; cell-mediated immunity, CMI; popliteal lymph node, PLN; Dinitrochlorobenzene, DNCB; intradermally, ID; maleic

vinyl ether, MVE; colony forming units, CFU; tumor necrosis factor binding protein, TNFbp; rheumatoid arthritis, RA; bovine serum albumin, BSA; polyethylene glycol, PEG; interleukin-1, IL-1; interleukin-1 receptor antagonist, IL-1RA; keyhole limpet hemocyanin, KLH; enzyme linked immunosorbent assay, ELISA; mouse ear swelling test, MEST; mixed lymphocyte response, MLR; reticulo-endothelial system, RES; polymorphonucleocyte, PMN;

Present Addresses: Corresponding author: DB Colagiovanni, Gilead Sciences, Inc. 2860 Wilderness Place, Boulder, CO 80301; M Suniga, Baxter. 2545 Central Ave, Boulder CO 80301, GM Shopp, Elan Pharmaceuticals, 800 Gateway Blvd. So. San Francisco, CA 94080

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